COD/sulfate ratio does not affect the methane yield and microbial diversity in anaerobic digesters

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ABSTRACT

Anaerobic digestion of organic matter is the major route of biomethane production. However, in the presence of sulfate, sulfate-reducing bacteria (SRB) typically outcompete methanogens, which may reduce or even preclude methane production from sulfate-containing wastewaters. Although sulfate-reduction and methanogenesis can occur simultaneously, our limited understanding of the microbiology of anaerobic digesters treating sulfate-containing wastewaters constrains improvements in the production of methane from these systems. This study tested the effects of carbon sources and chemical oxygen demand-to-sulfate ratio (COD/SO_4^{2-}) on the diversity and interactions of SRB and methanogens in an anaerobic digester treating a high-sulfate waste stream. Overall, the data showed that sulfate removal and methane generation occured in varying efficiencies and the carbon source had limited effect on the methane yield. Importantly, the results demonstrated that methanogenic and SRB diversities were only affected by the carbon source and not by the COD/SO_4^{2-} ratio.

Keywords: Anaerobic digestion, methanogens, sulfate reducers, COD/sulfate ratio

1. INTRODUCTION

Anaerobic digestion (AD) has been successfully deployed for decades to treat high-strength industrial wastewaters and sewage sludge. Since methane, a renewable energy source, is generated as the major end product, AD is considered the most sustainable treatment process with a global primary energy potential of 99 EJ/year projected for 2050 (Koornneef et al., 2013). However, the most recent estimates indicate that currently only around 2.1 EJ/year is produced from the anaerobic digestion of waste (WBA, 2014). Efficient AD process (from complex organic matter degradation to biomethane generation) requires the concerted action of a well-balanced microbial consortium composed of hydrolysers, fermenters, syntrophic microorganisms and methanogens. Despite numerous studies characterising these key players, many unidentified microorganisms and unresolved metabolic pathways are regularly observed in AD reactors, hence the AD process is still considered a ‘black-box’ (Schmidt et al., 2016).

While many high-strength industrial wastewaters can be treated efficiently via anaerobic digestion, anaerobic treatment of sulfate-containing wastewaters, such as from the brewery, pulp and paper, food processing, and tannery industries, generates very little methane. In sulfate-containing wastewaters terminal oxidation occurs via both sulfate reduction and methanogenesis. Sulfate-reducing bacteria (SRB) use sulfate as their terminal electron acceptor and can outcompete methanogenic archaea for carbon and electrons (O'Flaherty et al., 1998). SRB may also compete with syntrophic bacteria (e.g. acetogens) for short-chain volatile fatty acids such as propionate and butyrate (Qatibi et al., 1990), while hydrogen sulfide production by SRB can inhibit both methanogens and SRB (O'Reilly and Colleran, 2006). In addition to the competitive interaction between methanogenic archaea and SRB, co-existence of methanogenesis and sulfate reduction has been demonstrated in different ecosystems with high sulfate concentrations such as estuarine sediments (Oremland and Polcin, 1982) and anaerobic digesters (Isa et al., 1986). In environments with low sulfate concentrations, H_2-utilising methanogens scavenge hydrogen produced during acidogenesis and provide energetically favourable conditions for syntrophic SRB or acetogens (Parkin et al., 1990; Muyzer and Stams, 2008; Bae et al., 2015). Moreover, the flexible metabolism of many SRB increases their chance of survival in the absence of sulfate (Plugge et al., 2011).

The interaction between methanogens and SRB is governed by several factors such as the type and oxidation state of organic carbon as well as the carbon-to-sulfate ratio (Bhattacharya et al., 1996; Raskin et al., 1996; Hu et al., 2015; Lu et al., 2016). For instance, it has been shown that SRB in natural sediments prefer simple organic compounds such as ethanol and acetate over more complex organic compounds and usually outcompete methanogens if sulfate is available (Oremland and Polcin, 1982; Pol et al., 1998). However, anaerobic metabolism in high-rate engineered systems such as anaerobic digesters may differ significantly from natural sediments. In anaerobic reactors treating sulfate-containing wastewaters, the carbon (measured as chemical oxygen demand, COD) to sulfate ratio (COD/SO_4^{2-}) has been found to be critical in determining the fate of the carbon; this ratio is usually kept above the theoretical value of 0.67 to ensure complete sulfate removal. However, results from previous research on the effect of COD/SO_4^{2-} are contradictory. For instance, methane production from an upflow anaerobic sludge bed (UASB) reactor greatly deteriorated when the COD/SO_4^{2-} ratio fell below 2 (Choi and Rim, 1991; Lu et al., 2016), whilst other studies did not observe a significant effect of sulfate on methanogenesis (Hoeks et al., 1984; Hu et al., 2015). The inconsistency between these observations may be due to the differences in operational conditions such as wastewater characteristics and reactor type used. Our knowledge of the diversity and metabolism of microorganisms in AD reactors receiving sulfate-containing wastewaters is still very limited, which restricts our understanding of these systems and hinders the development of strategies to improve the methane production from AD reactors. In particular, sulfate may affect the degradation pathway of carbon compounds present in the influent and of the associated volatile fatty acids. Therefore, the effect of the COD/SO_4^{2-} ratio on the interactions between SRB and methanogens as well as on the degradation pathway of carbon compounds needs to be addressed.

In this study, we systematically evaluated the impact of three different COD/SO_4^{2-} ratios and four different carbon sources on the methane yield and on the microbial population dynamics in anaerobic sludge samples collected from a full-scale anaerobic digester treating a sulfate-containing waste stream. Results revealed how the carbon source and COD/SO_4^{2-} ratio affected the methane yield, the interactions between SRB and methanogens and the metabolic pathways in anaerobic digester samples under sulfidogenic conditions previously considered as unfavourable for methane generation.

2. MATERIALS AND METHODS

2.1. Sample collection

Anaerobic sludge samples were collected in July 2015 from three different sampling ports of a UASB reactor of an industrial treatment plant that receives coffee production wastewater (Jacobs Douwe Egberts Ltd, Banbury, UK), which contains sulfate. So, the anaerobic sludge is acclimatised to sulfate. Samples were transferred to the laboratory immediately and kept at 4°C until the experiments were set up.

2.2. Potential methane production test

A potential methane production (PMP) test was conducted to determine the optimum concentrations and incubation times for four carbon sources (acetate, propionate, butyrate and trimethylamine) to maximise methane production. Acetate, propionate and butyrate were chosen as competitive, whilst trimethylamine (TMA) was chosen as a non-competitive substrate for methanogens. Sludge samples from the three sampling ports were mixed and washed twice in anaerobic medium with vitamin solution (DSMZ 318 and DSMZ 141, respectively; Braunschweig, Germany) to remove sulfate and organic compounds from the samples. The washed sludge was centrifuged at 4000 *g* for five minutes, the supernatant was decanted and the resulting pellet was resuspended in equal volume of anaerobic medium as the removed supernatant. Triplicate incubations were set up in 60 ml crimp-top serum bottles with 30 ml liquid volume. Seed sludge with 1000 mg/l volatile suspended solids (VSS) was added to the bottles. Acetate was tested at final concentrations of 10 to 60 mM, the other three carbon sources were tested at 10 to 25 mM. The bottles were closed with butyl-rubber stoppers and crimp-sealed with aluminium caps, flushed with oxygen-free nitrogen gas for 10 min and then incubated at 35°C with shaking (150 rpm, Innova 4300, New Brunswick Scientific Ltd., UK). Headspace gas pressure was measured daily using a handheld digital manometer (Dwyer Series 475, Dwyer Instruments Ltd, UK) and the incubations were ceased once gas production stopped.

PMP test results showed that the highest methane productions were obtained when the samples were incubated with 45 mM acetate, 20 mM propionate, 15 mM butyrate and 15 mM trimethylamine (Supplementary Figure 1). Incubations with acetate, propionate and butyrate reached the highest PMP on day seven whilst TMA incubations took 12 days. Therefore, experiments were set up using these concentrations and incubated for seven (acetate, propionate and butyrate) or 12 days (TMA) to provide conditions for maximum methane production and avoid substrate inhibition.

2.3. Experimental design

Batch experiments were used to assess the impact on methane yield of acetate, propionate, butyrate and TMA at three different COD/SO_4^{2-} ratios (0.5, 1.5 and 5) and to analyse interactions between anaerobic microbial populations. No-sulfate incubations were set up as controls. Five replicated microcosms were prepared for each substrate and COD/SO_4^{2-} combination using inoculum adjusted to 1000 mg/l VSS in 60 ml serum bottles with 30 ml liquid volume. Guided by the PMP test, different carbon (15, 20 or 45 mM) and sulfate (1.5 – 66.7 mM) concentrations were provided to establish the selected COD/SO_4^{2-} ratios (Supplementary Table 1). The microcosms were run for seven (acetate, butyrate and propionate) or 12 days (TMA).

2.4. Methane and volatile fatty acids analysis

At the end of the incubations, gas samples were collected using a gas-tight syringe (Hamilton Company, Reno, USA) and the methane production was monitored by gas chromatography (Agilent 6890N, Agilent Technologies, Cheshire, UK) fitted with a flame ionisation detector and Porapak Q column. Nitrogen with 20 ml/min was used as the carrier gas. Three measurements were taken for each microcosm and the mean was calculated.

Slurry samples were also collected at the end of the incubations and centrifuged at 4000 *g* for five minutes. Supernatant was collected, filtered through a 0.20 μm polyethersulfone membrane and analysed for volatile fatty acids (VFA) and sulfate using an ion exchange chromatography (Dionex ICS3000; Dionex Corp., Sunnyvale, CA, USA). Anion analysis was done using an Ionpac AS 18 column (2 mm x 50 mm) equipped with an Ionpac AS 18 guard column, while cation analysis was done using an Ionpac CS12A column (4 mm x 250 mm) equipped with an Ionpac CG12A guard column. A gradient of 0-30 mM KOH and 20 mM methylsulfonic acid was used as eluent for anion and cation analyses, respectively.

2.5. Molecular methods

2.5.1. DNA extraction and PCR

Three replicates (out of five) that had less than 5% difference in methane generation from each treatment and controls were chosen for molecular analysis. Slurry samples were collected as above and total genomic DNA was extracted from 500 mg of centrifuged slurry from each selected incubation using the hydroxyapatite spin-column method (Purdy, 2005). Bacterial and archaeal 16S rRNA genes and functional genes specific to methanogens (methyl coenzyme M reductase, *mcrA*) and SRB (dissimilatory sulfate reductase, *dsrB*) were amplified by PCR (Supplementary Table 2). All PCR amplifications were carried out using a Mastercycler Pro thermal cycler (Eppendorf UK Ltd., Stevenage, UK) with MyTaq Red DNA Polymerase (Bioline Reagents Ltd., London, UK). Amplification conditions for the 16S rRNA and the *mcrA* genes were as follows: initial denaturation at 95°C for 5 min, 35 cycles of 95°C for 1min, 55°C for 1 min, 72°C for 1.5 min, a final elongation step at 72°C for 5 min. For the *dsrB* gene, the PCR conditions were the same except the annealing temperature, which was 52°C.

2.5.2. *High-throughput sequencing and data analysis*

16S rRNA and functional gene PCR products were sequenced on the Illumina MiSeq platform (300 bp paired-end, Illumina, Inc, San Diego, CA, USA) at the University of Warwick (UK). Before sequencing, the PCR products were cleaned using Charge Switch PCR Clean-up kit (Invitrogen, CA, USA), quantified by Qubit dsDNA BR Assay Kit with Qubit 2.0 Fluorometer (Invitrogen, CA, USA), and prepared for sequencing as described by Caporaso et al. (2012).

We obtained 4.7, 3.3, 8.6 and 7.5 Gb raw sequences for the *mcrA*, *dsrB*, bacterial and archaeal 16S rRNA genes, respectively.   Raw sequences were quality-trimmed using Trimmomatic (Bolger et al., 2014). Merging and operational taxonomic unit (OTU) picking were carried out by USEARCH v8 (Edgar, 2010) at 97% and 85% similarity cut-off for the 16S rRNA and the functional gene sequences, respectively. Chimeras were checked using ChimeraSlayer (Haas et al., 2011) and removed from downstream analysis. Taxonomy assignments were determined against the Greengenes database (DeSantis et al., 2006) for bacteria and archaea, and custom *dsrB* and *mcrA* databases (Müller et al., 2015; Wilkins et al., 2015) using RDP Classifier 2.2 (Wang et al., 2007) via QIIME software, version 1.6.0 (Caporaso et al., 2010). Average relative abundance for each OTU in the samples was calculated using the relative OTU read abundances of three replicates.Sequence datasets have been submitted to the National Center for Biotechnology Information (NCBI) Read Archive under the bioproject accession number of PRJNA434657.

2.5.3. Quantitative PCR (qPCR)

In order to relate the methane generation to the relative abundance of methanogens, total *mcrA* gene copies in the incubation bottles were quantified using a qPCR assay with the *mcrA*-specific PCR primers (Supplementary Table 2). A standard curve was produced using serial 10-fold dilutions of a plasmid containing the *mcrA* gene. PCR reaction volumes were 10 ul, comprising 2 ul of 1:10 diluted gDNA, 0.35 ul of each primer, 2.3 ul H_20 and 5 ul SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories Ltd., Hertfordshire, UK). Samples were run on a Bio-Rad CFX Connect Real-Time Detection System (Bio-Rad Laboratories Ltd., Hertfordshire, UK). The cycling conditions were as follows: 98 °C for 3 min, followed by 40 cycles of 98 °C for 15 s, 55 °C for 15 s, 72 °C for 1 min. To check for non-specific DNA products, a melt curve was performed by heating the reaction mixture from 65 to 95°C with 0.5°C increments. The efficiency of the reactions was between 103%-109%, while the R^2 value for the standard curve was 96%.

2.6. Statistical analysis

One-way ANOVA with Post-hoc Dunnett's test was conducted to determine the statistical significance of difference in biomethane production in the microcosms. Species richness (Chao1) and alpha diversity (Shannon's index) were calculated using OTU numbers and relative abundances. Principal Components Analysis (PCA) was also applied to the relative abundance of OTUs to discriminate the samples with respect to treatments. Following this, Spearman's correlation analysis was carried out to identify the factors that may have affected the OTU abundances by correlating the first two principal components to the experimental variables including the methane generation, sulfate removal efficiency, COD/SO_4^{2-} ratio as well as the concentrations of sulfate and the carbon compound removed. Graphpad Prism 7 software (Graphpad Software, CA, USA) was used for correlation analysis and one-way ANOVA test, while PAST (version 3) was used for diversity indices and PCA (Hammer et al., 2001).

3. RESULTS AND DISCUSSION

3.1. Methane production and sulfate reduction efficiencies under different COD/SO_4^{2-} ratios

Methane, VFA and sulfate concentrations in the microcosms were measured at the end of the incubation and mass balances were calculated (Table 1). Results showed that three carbon sources (acetate, propionate and butyrate), and any VFAs produced as by-products, were consumed during the incubation period (data not shown). However, ~30% of the added TMA (123 to 137 μmoles) was not consumed in the incubation time.

The methane production and sulfate reduction for each substrate were assessed by comparison to the no-sulfate control microcosms. Methane production was also compared to the theoretical methane yields based on the amount of substrate utilised (Bushwell and Mueller, 1952). Both acetate- and propionate-amended microcosms produced methane in amounts close to their theoretical maximum (1350 μmoles and 1050 μmoles, respectively, Figure 1a, Table 1), while butyrate- and TMA-amended microcosms produced no more than 60% of their theoretical maximum (1500 μmoles and 704-734 μmoles, respectively, Figure 1a, Table 1).

In acetate-amended microcosms, there was no significant difference between the methane generation in controls and sulfate-amended microcosms. Similarly, in a previous study, an anaerobic sludge sample, acclimated to sulfate-rich pulp and paper wastewater, utilized 2000 mg/L acetate and produced 700 mL methane, which was approximately the theoretical maximum (Ince et al., 2007). On the other hand, propionate and TMA had lower methane yields when COD/SO_4^{2-} ratio was 0.5 and 1.5 compared to the controls, while butyrate-amended samples with all COD/SO_4^{2-} ratios had lower methane yields compared to the controls. It should be noted that hydrogen sulfide (H_2S) produced by the reduction of sulfate might have an inhibitory effect on some methanogenic species, which might lower the methane generation (Isa et al., 1986). However, we used sulfate-acclimated anaerobic sludge to set up the experiments, so the inhibitory effect of H_2S would likely be reduced in our microcosms.

This may be the reason why we did not observe any significant drop in methane generation from acetate-amended microcosms with or without sulfate. Furthermore, there is experimental evidence that the kinetic and thermodynamic advatages of sulfate reducers over methanogens are erased by their sensitivity to sulfide toxicity, which may explain the methanogenic activity observed in our microcosms amended with sulfate (Maillacheruvu and Parkin, 1996).

The effect of COD/SO_4^{2-} ratio on methane generation and sulfate removal in the microcosms was limited, and depended on the carbon source utilised. There was no significant effect of changing the COD/SO_4^{2-} ratio on the methane production in the acetate-, butyrate- and TMA-amended samples (Figure 1a, $p>0.05$). There was a small, but significant ($p<0.01$) decrease in methane production in propionate-amended samples at a COD/SO_4^{2-} ratio of 1.5. However, even within these microcosms there was no pattern of decreasing methane production with increasing sulfate. The consistent methane production with an increasing COD/SO_4^{2-} ratio suggests that sulfate reduction does not affect methanogenesis in either the acetate- or propionate-amended microcosms. This is despite the fact that, in other systems, both of these substrates are preferentially utilised by SRB if sulfate is freely available (Purdy et al., 2003a, 2003b) and acetate-based sulfate reduction is more thermodynamically favourable than acetoclastic methanogenesis (Schönheit et al., 1982).

Methane production in the butyrate- and TMA-amended microcosms was between 44% - 82% of their theoretical maximum (Figure 1a, Table 1) in all treatments and significantly lower than the no-sulfate controls in all samples. However, there was no significant difference in methane production across the three COD/SO_4^{2-} ratios for both substrates, which suggests that the presence but not the concentration of sulfate affected the methane production. The limited methane production with butyrate and

TMA indicates that non-methanogenic pathways for both butyrate and TMA degradation occurred in these incubations.

Sulfate removal efficiency increased with increasing COD/SO_4^{2-} ratio in all four treatments (Figure 1b, Table 1) with only the acetate-amended microcosms not reaching ~100% removal of sulfate (maximum of 70% removal). The effect of sulfate addition on methane production in TMA-amended microcosms is remarkable, as this compound is not known to be a competitive substrate for SRB. Hence, our results disagree with those of Vich et al (2011), who amended methylamine and sulfate to sludge samples from a full-scale UASB reactor and observed no significant effect of sulfate addition on methane generation. In the propionate-amended samples at 0.5 and 1.5 COD/SO_4^{2-} ratios, available sulfate had a small but statistically significant effect on methane production ($p<0.01$; Figures 1a and 1b). Our results contradict two recent studies, where the effect COD/SO_4^{2-} ratio on methane generation was investigated. In a study by Lu et al. (2016) on the effect of influent COD/SO_4^{2-} ratio on the biodegradation of starch wastewater in a lab-scale UASB reactor, sulfate addition enhanced sulfidogenesis and subsequently methanogenesis. However, when the COD/SO_4^{2-} ratio was lower than 2, methanogenesis was supressed, possibly due to the competition and H_2S inhibition (Lu et al., 2016). Similarly, Kiyuna et al. (2017) found that high sulfate concentrations significantly reduced methane production from sugarcane vinasse, however these authors used higher COD/SO_4^{2-} ratios (7.5, 10 and 12) than we used in our study.

While our results showed that methane production and sulfate reduction are independent pathways for readily biodegradable substrates, in full-scale applications, both COD removal efficiency and methane production in anaerobic treatment of complex, sulfate-rich wastewaters may be lower. This may be due to the low biodegradability of wastewater and the inhibitory effect of high sulfate/sulfur concentration on microbial activity (Lens et al., 1998).

3.2. Taxonomic and functional diversities in the microcosms

Between 1.7 and 3.8 million quality-filtered, chimera-free sequences were obtained for bacterial 16S rRNA, archaeal 16S rRNA, *dsrB* and *mcrA* genes. These sequences were assigned to 1295 and 543 distinct OTUs at 97% identity for bacterial and archaeal 16S rRNA genes, whilst 288 and 61 distinct OTUs were obtained at 85% identity for *dsrB* and *mcrA* genes, respectively. There was no significant difference between the observed and predicted numbers of OTUs for each marker gene within each treatment as estimated by Chao1 (Supplementary Table 3). The Shannon diversity index did not vary significantly across samples (Supplementary Table 3).

We observed distinct shifts in the specific microbial populations in the microcosms over the experimental period, which allowed us to draw conclusions about the impact of carbon sources and the COD/SO_4^{2-} ratio on the diversity and metabolic interactions of SRB and methanogens.

3.2.1. Methanogenic diversity and abundance

Methanobacterium spp, which use H_2 and CO_2 to produce methane (Boone, 2001), dominated the methanogenic communities in all incubations (67-82% of the *mcrA* sequences, Figure 2a). This finding was confirmed by archaeal 16S rRNA sequencing (Figure 3a). The strong dominance of hydrogenotrophic methanogens even in the presence of sulfate demonstrates that H_2-consuming methanogens were not outcompeted by H_2-consuming SRB, which has been suggested to be a characteristic of nutritious, high-rate systems such as anaerobic digesters (Ueki et al., 1992). The consistently low percentage of *Methanosaeta* sequences (0.1-0.7%) in all the microcosms indicates that acetoclastic methanogenesis was not a significant process in this bioreactor (Demirel and Scherer, 2008). This is clearly shown in the fact that even the addition of acetate did not enhance *Methanosaeta* (Figure 2a), suggesting acetoclastic methanogenesis was not active at all in these slurries, despite the fact that 100% of the predicted methane was produced in the acetate-amended samples (Figure 1a).

In the TMA-amended microcosms, the methanogenic community structure shifted. In these incubations, the relative abundance of the obligate methylotrophic genus *Methanomethylovorans* (Lomans et al., 1999) increased significantly to 20.1% (±1.8%, p=0.003) from 1% in the other incubations, irrespective of the COD/SO_4^{2-} ratio (Figure 2a). Methylotrophic methanogens dominate TMA degradation in marine sediments (King, 1984; Purdy et al., 2003a), so it is not unexpected that sulfate reduction and methanogenesis were independent in TMA-amended microcosms and the relative abundance of *Methanomethylovorans* was not affected by the presence or the concentration of sulfate (Figure 2a). PCA analysis of the *mcrA* sequences also supported this finding, as it separated the TMA incubations from the rest of the samples (Figure 2b). The first principal components explained 82% and 84% of the total variability in the *mcrA* (Figure 2b) and archaeal diversities (Figure 3b) in the samples, respectively.

In addition to the sequence analysis, we have also quantified the *mcrA* genes to reveal the abundance of the methanogens in the samples. The *mcrA* gene numbers increased about ten-fold, from about 1.1×10^5 to about 1.6×10^6 across all microcosms (Supplementary Figure 2). The pattern was different for different substrates, though. The average abundance of the methanogens increased from 2.3×10^5 to 1.6×10^6 in the acetate-amended microcosms as the COD/SO_4^{2-} ratio increased, however this increase was not statistically significant. There was also no statistically significant difference in the methanogen abundance in propionate-amended microcosms, in spite of an increase in methane production at the highest COD/SO_4^{2-} ratio. This suggests an increase in the specific methanogenic activity in these microcosms. The lowest methanogen abundance was observed in the butyrate-amended microcosms, which was consistent with the methane production in these incubations, where the methane yield was lower than the other microcosms (Figure 1). The number of methanogens did not change significantly in the TMA-amended microcosms and they had a similar number of methanogens to acetate and propionate incubations although the methane yield was lower. This might be due to the lower efficiency of *Methanomethylovorans* spp in utilising TMA compared to hydrogenotrophic methanogens dominating other incubations.

Correlation analyses revealed that the *mcrA* and archaeal diversities did not significantly correlate with the COD/SO_4^{2-} ratio in the microcosms, while the first principal component of the *mcrA* analysis significantly correlated with only the methane yield ($p < 0.01$; Figure 2b and 3b; Table 2). Methanogen abundance did not correlate significantly with the methane yield in the microcosms, however sulfate removed was significantly related to the archaeal diversity (Table 2).

3.2.2. SRB diversity

The SRB diversity, as determined by sequencing the *dsrB* gene, did not change markedly with the COD/SO_4^{2-} ratio in the microcosms (Figure 4a). This counterintuitive result could be explained by the metabolic flexibility of SRB, which allows some of them act as fermenters when sulfate is not available (Plugge et al., 2011). Some SRB can form syntrophic associations with H_2 scavengers such as hydrogenotrophic methanogens, utilising the H_2 produced by SRB (Bryant et al., 1967; Stams and

Plugge, 2009). Indeed, hydrogenotrophic methanogenesis was the dominant methanogenic pathway in the microcosms, which might enable SRB survival in the sulfate-free control incubations.

The relative mean read abundance of the *Desulfarculus baarsii* lineage increased from ~6% to 14%-23.5% in the butyrate-amended microcosms (Figure 4a). *Desulfarculus baarsii* can oxidise acetate and fatty acids completely to CO_2 using sulfate as an electron acceptor (Sun et al., 2010). Although they have not been shown to grow without sulfate in syntrophy with methanogens to date (Muyzer and Stams, 2008; Plugge et al., 2011), they were found in the control incubations without added sulfate. However, presence does not mean activity: these *D. baarsii* species may have been present but inactive in the control incubations without sulfate. PCA analysis of the *dsrB* sequence data revealed that the first component accounted for 97.9% of the total variability, separating the butyrate incubations from the rest of the samples (Figure 4b). Interestingly, there was no significant correlation between the *mcrA* and *dsrB* diversities, and with the COD/SO_4^{2-} ratio (Table 2), which further indicates that methane production and sulfate reduction were independent processes in these samples. However, the *dsrB* diversity was found to be correlated with the concentration of sulfate removed, sulfate removal efficiency, the initial carbon concentration and the methane yield (Table 2).

3.2.3. Bacterial Diversity

The most striking result from the bacterial sequence analysis was the dramatic increase in the relative abundance of the genus *Syntrophomonas* in the butyrate incubations to 8.9%± 1.02% from 1.1% ± 0.3% in the other microcosms (p=0.003, Figure 5a). As in the *mcrA* and *dsrB* diversities, this change was not dependent on the COD/SO_4^{2-} ratio (Figure 5a and 5b). *Syntrophomonas* species can degrade butyrate to acetate and H_2 (Schmidt et al., 2013), and have been shown to form syntrophic interactions with hydrogenotrophic *Methanobacterium* spp (Sousa et al., 2007). We suggest that the members of this genus worked in syntrophy with *Methanobacterium* spp., which utilised H_2 to produce methane, particularly in the butyrate-amended microcosms. Similar cooperation was observed in co-cultures of *Syntrophomonas wolfei* and *Methanospirillum hungatei*, which coupled butyrate degradation to acetate and H_2 formation during growth on butyrate (Schmidt et al., 2013).

All the microcosms, including the controls, consistently contained *Syntrophobacter* in relatively high abundances (3.6-7%). This is in line with a previous research, showing that *Syntrophobacterales* are a stable and resilient functional group of bacteria in anaerobic digestion systems (Werner et al., 2011). *Syntrophobacter* species can grow on acetate, propionate and butyrate, either by sulfate reduction or, in the absence of sulfate, by fermentation in syntrophy with methanogens and other H_2/formate oxidisers (Sobieraj and Boone, 2006; Müller et al., 2010, 2013) . Their metabolic flexibility may explain their high relative abundance across the samples regardless of the carbon compound or the COD/SO_4^{2-} ratio used (Boone and Bryant, 1980; Muyzer and Stams, 2008; Plugge et al., 2011).

Bacterial diversity significantly correlated only with methane production ($p < 0.05$, Table 2), which may be due to the effect of the carbon sources on the bacterial populations, as clearly observed in the butyrate set. There was no significant correlation between bacterial diversity and COD/SO_4^{2-} ratio across the samples (Table 2).

3.3. Metabolic interactions between the microbial communities

Sulfate reduction and methane generation were observed in varying efficiencies in the microcosms, whilst the relative abundances of specific functional groups such as syntrophic organisms and hydrogenotrophic or methylotrophic methanogens (*Methanobacterium* spp and *Methanomethylovorans* spp.) did not vary considerably within each set despite the change in the COD/SO_4^{2-} ratio (Figure 2-5). This may be explained by the flexible metabolism of SRB, which allows these populations to survive when there is no available sulfate to respire as discussed above for *D.baarsii*. Furthermore, syntrophic associations between methanogens and SRB may have facilitated their growth together, as was previously shown in sulfate-amended anaerobic reactors, which had high sulfate-reduction efficiency even when hydrogenotrophic methanogens were dominant (Yang et al., 2015).

We derived metabolic pathways for the metabolism of the carbon compounds used in this study based on the dominant microbial populations as obtained by the sequence analysis. In acetate amended microcosms, efficient methane generation was observed with and without sulfate and there was no marked change in microbial diversity under different COD/SO_4^{2-} ratios. According to the sequence analysis, different metabolic pathways for the mineralization of acetate could be active simultaneously in these microcosms, independent of the COD/SO_4^{2-} ratio (Figure 6a).

Desulfarculus baarsii species can convert acetate to CO_2, which can be further used to produce methane. Similarly, syntrophic acetate oxidation coupled to hydrogenotrophic methanogenesis, which is thermodynamically and physiologically feasible at mesophilic temperatures, may have occurred efficiently in these microcosms (Schnürer and Nordberg, 2008; Dolfing, 2014). We propose that methane generation from propionate was via similar pathways (Figure 6b), with propionate being converted to acetate first as it is not utilised by methanogens directly. The dominance of the members of the *Desulfarculus baarsii* lineage and the genus *Syntrophobacter* suggests complete oxidation of propionate to H_2+CO_2 via acetate. Although propionate degradation to acetate is thermodynamically unfavourable under standard conditions ($\Delta G^{o'}$ = +76 kJ/mol), hydrogenotrophic methanogenesis in the microcosms could have lowered the H_2 partial pressure, providing suitable conditions for propionate conversion to acetate. Similar interactions were observed in paddy soils, where *Syntrophobacter* spp were found to be the dominant propionate degraders. These organisms were suggested to degrade propionate in synthrophy with hydrogenotrophic methanogens in the absence of sulfate, however they switch to sulfate reduction when sulfate became available (Liu and Conrad, 2017).

Metabolic pathways were different in butyrate and TMA-amended incubations as inferred from the bacterial and methanogenic community structures in these microcosms. Results suggest that the genus *Syntrophomonas* degraded butyrate to acetate. Meanwhile, members of the *Desulfarculus baarsii* lineage may have completely oxidised butyrate and produced CO_2 while reducing sulfate (Figure 6c). In the sulfate-free control incubations, they may have worked in syntrophy with H_2 oxidisers. Additionally, *Syntrophobacter* spp. likely degraded butyrate to CO_2 and H_2. Metagenomic analysis of samples from lab-scale anaerobic digesters demonstrated that *Syntrophobacterales* have the metabolic potential to degrade reduced carbon compounds such as butyrate and propionate to acetate, CO_2 and H_2 (Vanwonterghem et al., 2016). The highest relative abundance of *Syntrophobacter* spp (12% of *dsrB* sequences) was in the 0.5 COD/SO_4^{2-} ratio microcosms compared to 4.8-8% in control and higher COD/SO_4^{2-} ratios. The high abundance of these complete-oxidisers conflicts with findings of Muyzer and Stams (Muyzer and Stams, 2008), who suggested that incomplete oxidisers of SRB would dominate over complete oxidisers when degrading butyrate.

The increased relative abundance of the genus *Methanomethylovorans* in the TMA microcosms indicates that part of the TMA was converted to methane directly via methylotrophic methanogenesis (Figure 6d). Interestingly, sulfate removal was also observed in these incubations although TMA has not been shown to be a growth substrate for SRB previously. Interspecies H_2 transfer between *Methanomethylovorans* spp. and the SRB may well have been the mechanism behind the sulfate reduction observed. As demonstrated previously, when methylotrophic methanogens and hydrogenotrophic SRB are in the same environment, the methanogens produce H_2, which serves as the electron donor for hydrogenotrophic SRB via interspecies H_2 transfer (Phelps et al., 1985; Finke et al., 2007). On the other hand, *Methanobacterium* spp (hydrogenotrophic methanogens) used H_2+CO_2 to generate methane. Hence, together with the hydrogenotrophic SRB, they would have maintained low H_2 concentrations, thus facilitating the H_2 production by methylotrophic methanogens (Meuer et al., 2002). Finke et al. (2007) have suggested that this H_2 loss mechanism allows the methanogens to be active even when sulfate is available. Indeed, in our experiments, the availability of sulfate did not affect the methanogenic diversity. However, further experiments are required to confirm the metabolic interaction between SRB and methylotrophic methanogens when degrading TMA.

The results of this study should be useful to develop strategies to increase the methane yield from full-scale anaerobic digesters receiving sulfate-containing wastewaters. For instance, a two-stage anaerobic treatment may be operated to increase the acetate and propionate concentrations during the acidification step. Since we have demonstrated that the COD/SO_4^{2-} ratio does not affect the methane production when acetate and propionate are the carbon surces, a higher methane yield may be obtained in the second reactor than when a one-reactor strategy is followed. Moreover, the acidification reactor can be operated under alkaline conditions to increase the propionate production when the influent is a protein-rich wastewater.

4. CONCLUSION

Our results demonstrate that in a microbial community sourced from a sulfate acclimated reactor, methane production and sulfate reduction were independent processes and that the COD/SO_4^{2-} ratio did not affect the microbial community structure, although the presence of sulfate can result in a shift in the metabolic pathway to simultaneous methanogenesis and sulfate reduction. The main factor influencing the microbial community structure, and hence the metabolic pathways, was the carbon source. This indicates a more important role for the substrate in anaerobic reactors than merely the COD/SO_4^{2-} ratio, which was previously suggested to be the key parameter.

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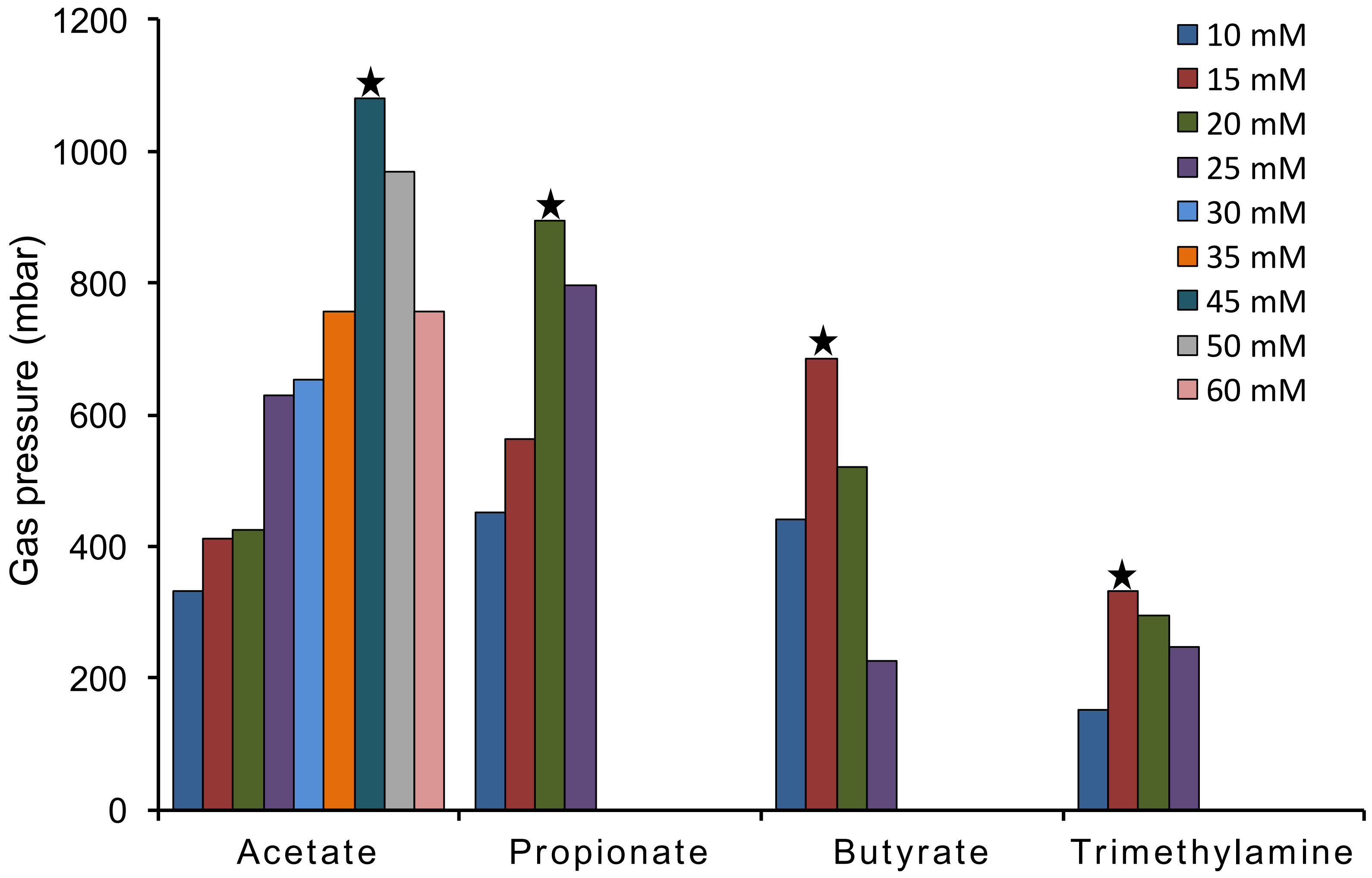
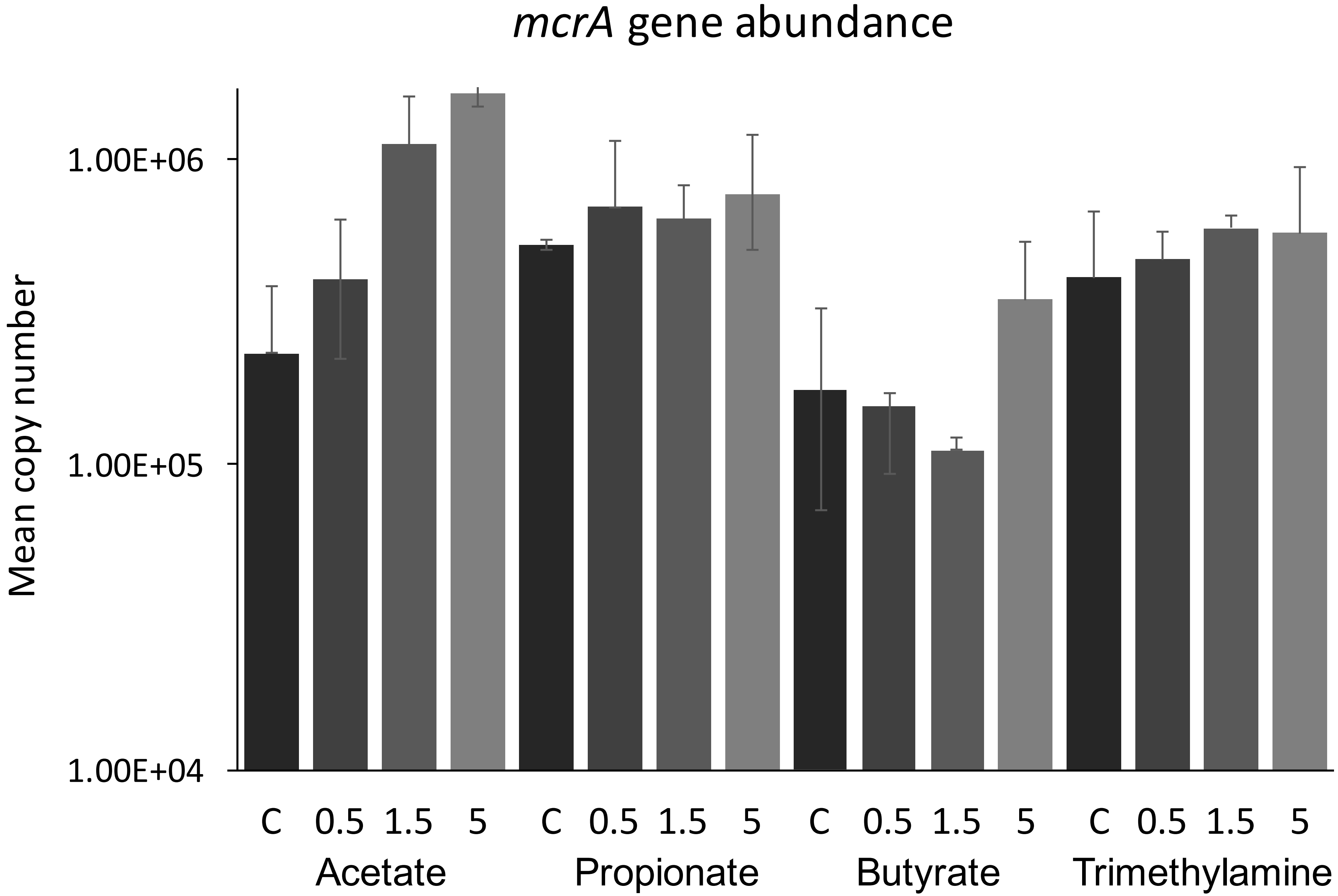
Supplementary Figures and Tables



Supplementary Figure 1. Biogas production during the specific methanogenic activity tests. Maximum gas production occurred at 45 mM acetate, 20 mM propionate, 15 mM butyrate or trimethylamine.



Supplementary Figure 2. Abundance of the *mcrA* gene in the incubations.

Supplementary Table 1. Experimental conditions used to set up the incubations.

Carbon source	Incubation	Substrate added (mM)	Sulfate added (mM)
Acetate	**Control**	45	0
	COD/SO4 = 0.5	45	59.7
	COD/SO4 = 1.5	45	19.9
	COD/SO4 = 5	45	5.9
Propionate	**Control**	20	0
	COD/SO4 = 0.5	20	46.5
	COD/SO4 = 1.5	20	15.5
	COD/SO4 = 5	20	4.6
Butyrate	**Control**	15	0
	COD/SO4 = 0.5	15	66.7
	COD/SO4 = 1.5	15	22.2
	COD/SO4 = 5	15	6.67
Trimethylamine	**Control**	15	0
	COD/SO4 = 0.5	15	15
	COD/SO4 = 1.5	15	5
	COD/SO4 = 5	15	1.5

Supplementary Table 2. Primers used in this study.

Target gene	**Primer name**	**Sequence (5'-3')**	**Reference**
16S rRNA Bacteria	515F	GTGCCAGCMGCCGCGGTAA	Caporaso et al., 2011
	806R	GGACTACHVGGGTWTCTAAT	
16S rRNA Archaea	Parch519F	CAGCCGCCGCGGTAA	Øvreås et al., 1997
	ARC915R	GTGCTCCCCCGCCAATTCCT	Stahl and Amann, 1991
dsrB	DSR1762Fmix	see Ref.	Pelikan et al., 2016
	DSR2107Rmix		
mcrA	mcrIRD -F	TWYGACCARATMTGGYT	Lever and Teske, 2015
	mcrIRD -R	ACRTTCATBGCRTARTT	

Supplementary Table 3. Alpha diversity indices calculated for each sample. C: No-sulfate control. 0.5, 1,5 and 5 represent COD/SO_4^{2-} ratios.

		Acetate				Propionate				Butyrate				TMA			
		C	0.5	1.5	5	C	0.5	1.5	5	C	0.5	1.5	5	C	0.5	1.5	5
Bacteria	OTU number	1070	1083	1016	1043	985	978	978	1029	926	880	953	884	1024	959	948	932
	Shannon's index	4.27	4.29	4.35	4.36	4.18	4.27	4.10	4.35	4.21	4.13	4.18	4.16	4.31	4.25	4.26	4.22
	Chao1	1362	1259	1186	1217	1221	1221	1173	1234	1149	1111	1209	1079	1213	1147	1121	1109
Archaea	OTU number	528	482	502	505	500	484	510	516	474	448	458	452	476	478	492	455
	Shannon's index	3.07	3.13	2.81	2.95	2.83	3.02	3.06	2.92	2.77	2.91	2.79	2.89	2.93	2.93	3.13	2.96
	Chao1	581	529	522.0	525	519	529	546	538	505	534	503	515	525	515	534	493
dsrB	OTU number	247	253	239	256	238	245	258	254	259	259	259	251	258	255	232	230
	Shannon's index	2.44	2.63	2.42	2.41	2.35	2.26	2.50	2.53	2.69	3.27	3.03	3.11	2.60	2.69	2.47	2.24
	Chao1	267	263	255	269	271	272	300	259	269	274	275	277	289	281	262	245
mcrA	OTU number	45	61	58	56	42	43	54	45	61	59	53	44	43	43	43	43
	Shannon's index	1.72	1.51	1.45	1.48	1.94	1.62	1.73	1.35	1.73	1.75	1.77	1.63	1.97	1.85	1.60	1.78
	Chao1	46	61	62	63	42	43	57	48	61	60	55	44	43	43	43	43

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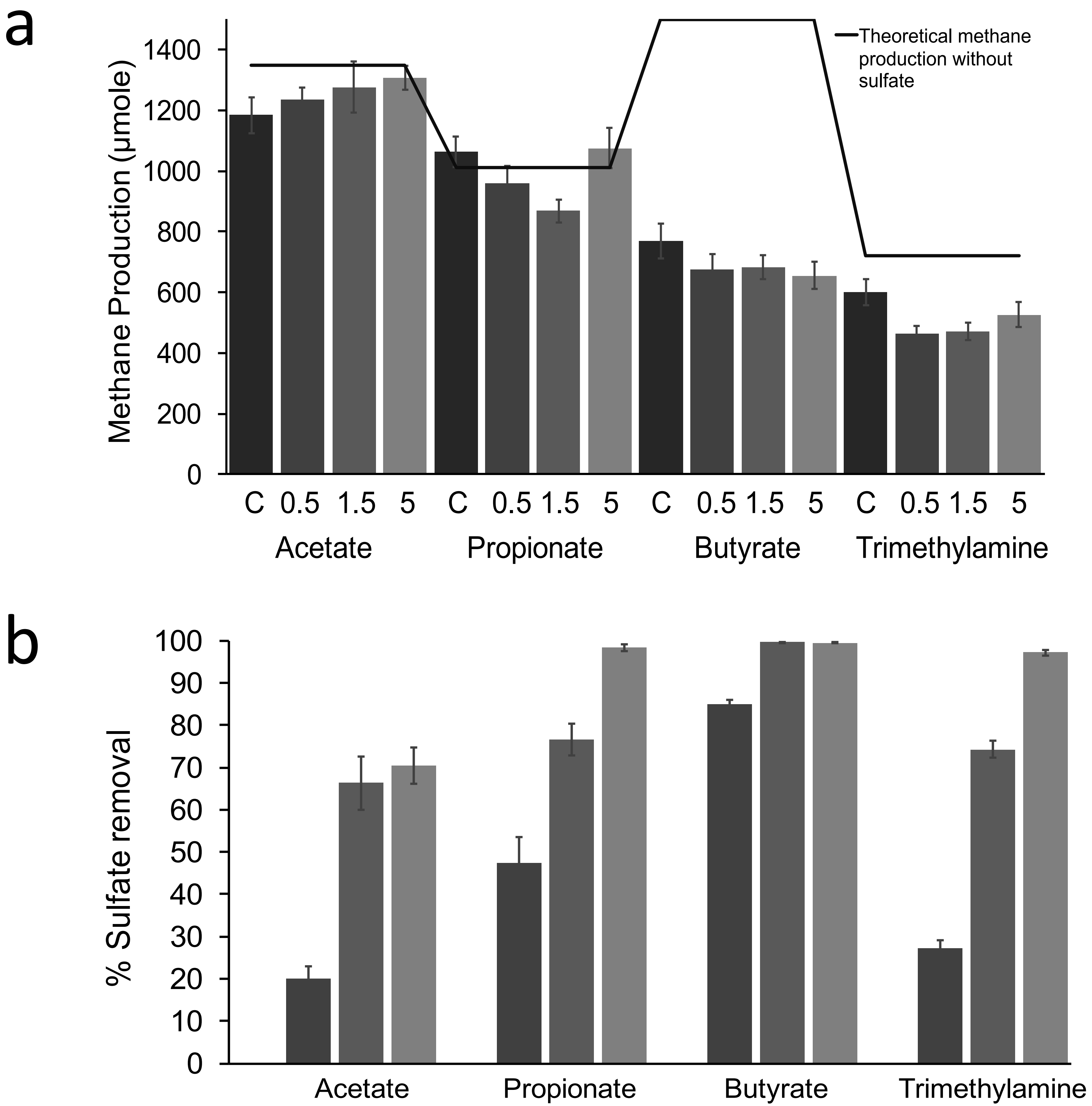
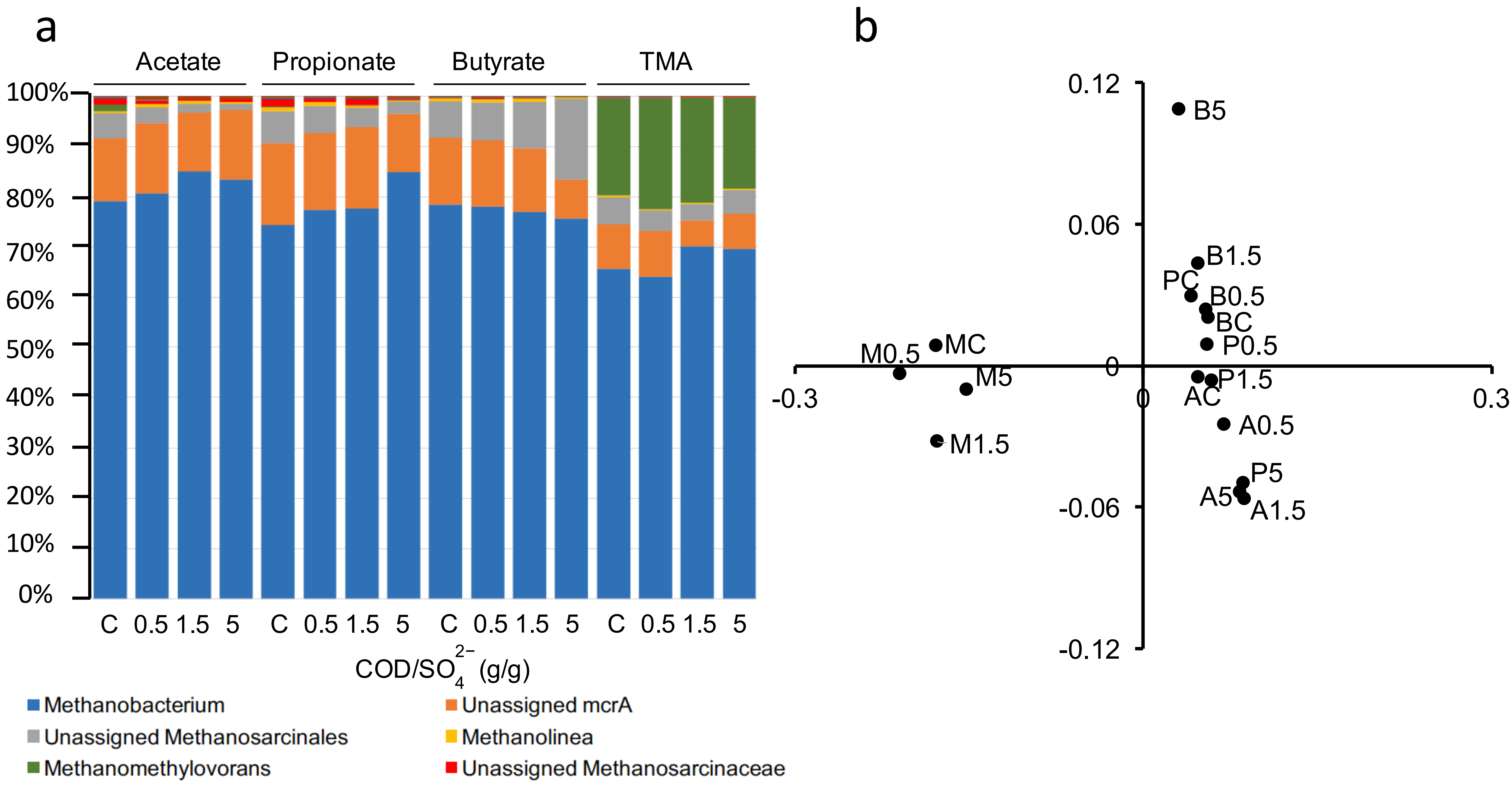
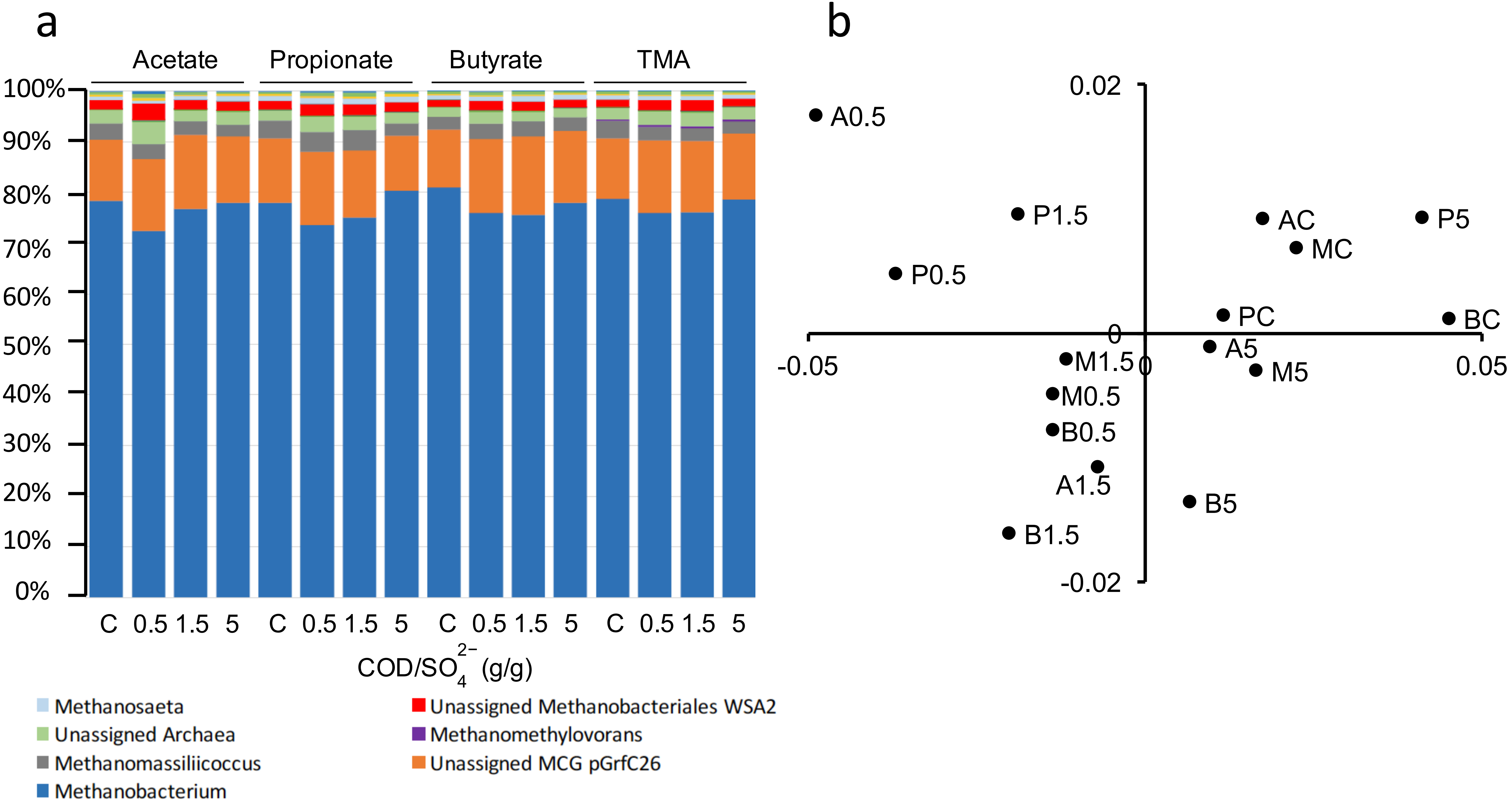
Table 1.

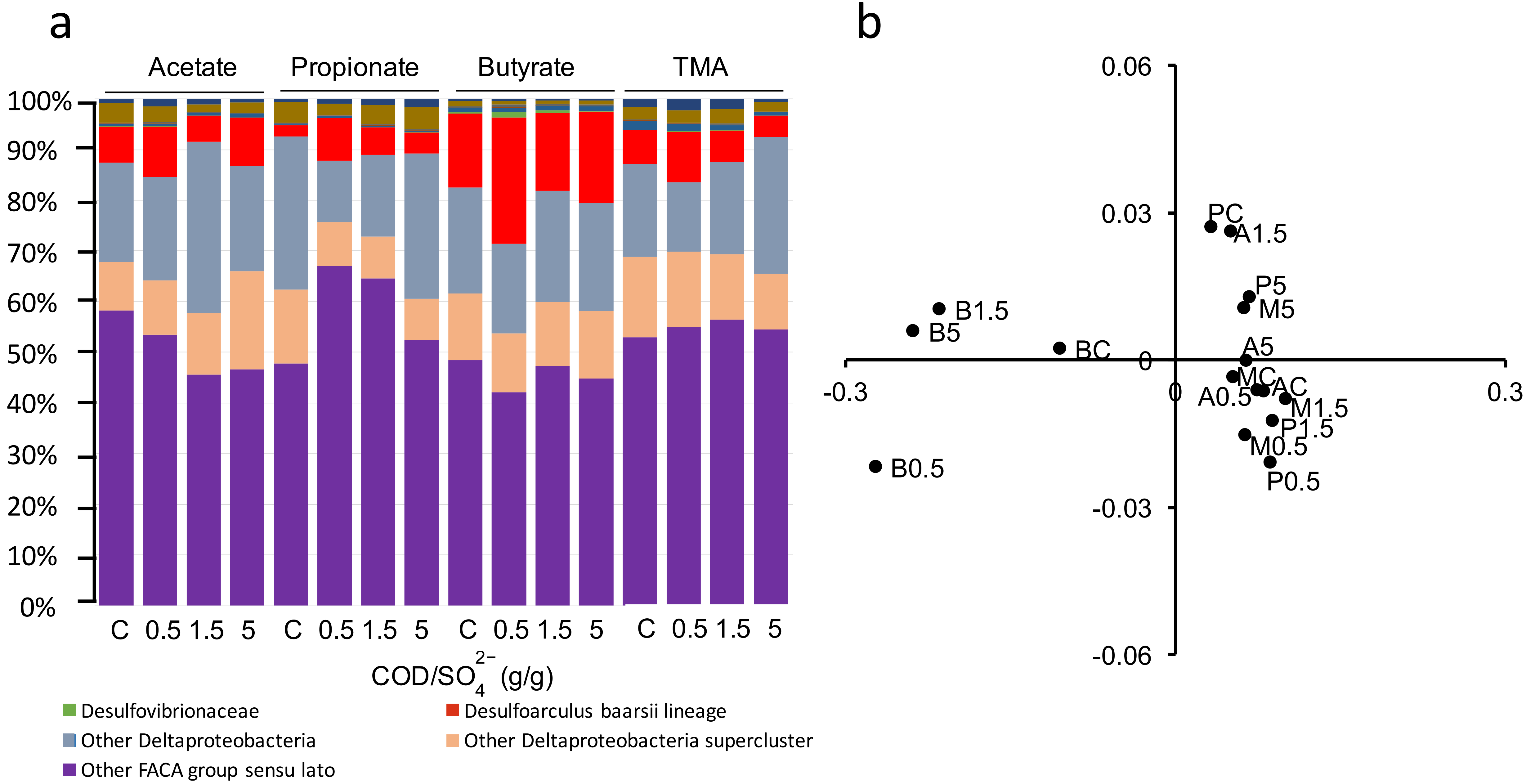
		Substrate			Methane yield			Sulfate		
	Treatment	Added	Residual	Consumed	Actual	Theoretical		Added	Consumed	
			µmol		µmol	µmol	%		µmol	%
Acetate 45 mM	Control	1350	0	1350	1184±117	1350	88	0	0	--
	COD/SO_4^{2-} = 0.5	1350	0	1350	1237±73	1350	92	1792	358	20±6
	COD/SO_4^{2-} = 1.5	1350	0	1350	1276±169	1350	95	597	377	66±16
	COD/SO_4^{2-} = 5	1350	0	1350	1307±78	1350	97	179	126	70±9
Propionate 20 mM	Control	600	0	600	1164±99	1050	111	0	0	--
	COD/SO_4^{2-} = 0.5	600	0	600	961±118	1050	92	1397	662	47±12
	COD/SO_4^{2-} = 1.5	600	0	600	869±74	1050	83	466	357	77±7
	COD/SO_4^{2-} = 5	600	0	600	1214±167	1050	116	140	138	99±1
Butyrate 15 mM	Control	600	0	600	770±117	1500	51	0	0	--
	COD/SO_4^{2-} = 0.5	600	0	600	677±98	1500	45	2002	1694	84±2
	COD/SO_4^{2-} = 1.5	600	0	600	683±76	1500	46	667	665	99±0.2
	COD/SO_4^{2-} = 5	600	0	600	656±90	1500	44	200	199	99±0.2
TMA 15 mM	Control	450	123	327	602±83	734*	83	0	0	--
	COD/SO_4^{2-} = 0.5	450	137	313	466±43	704*	66	451	112	25±4
	COD/SO_4^{2-} = 1.5	450	130	220	473±58	720*	66	150	113	74±4
	COD/SO_4^{2-} = 5	450	127	323	527±79	727*	73	45	44	98±1

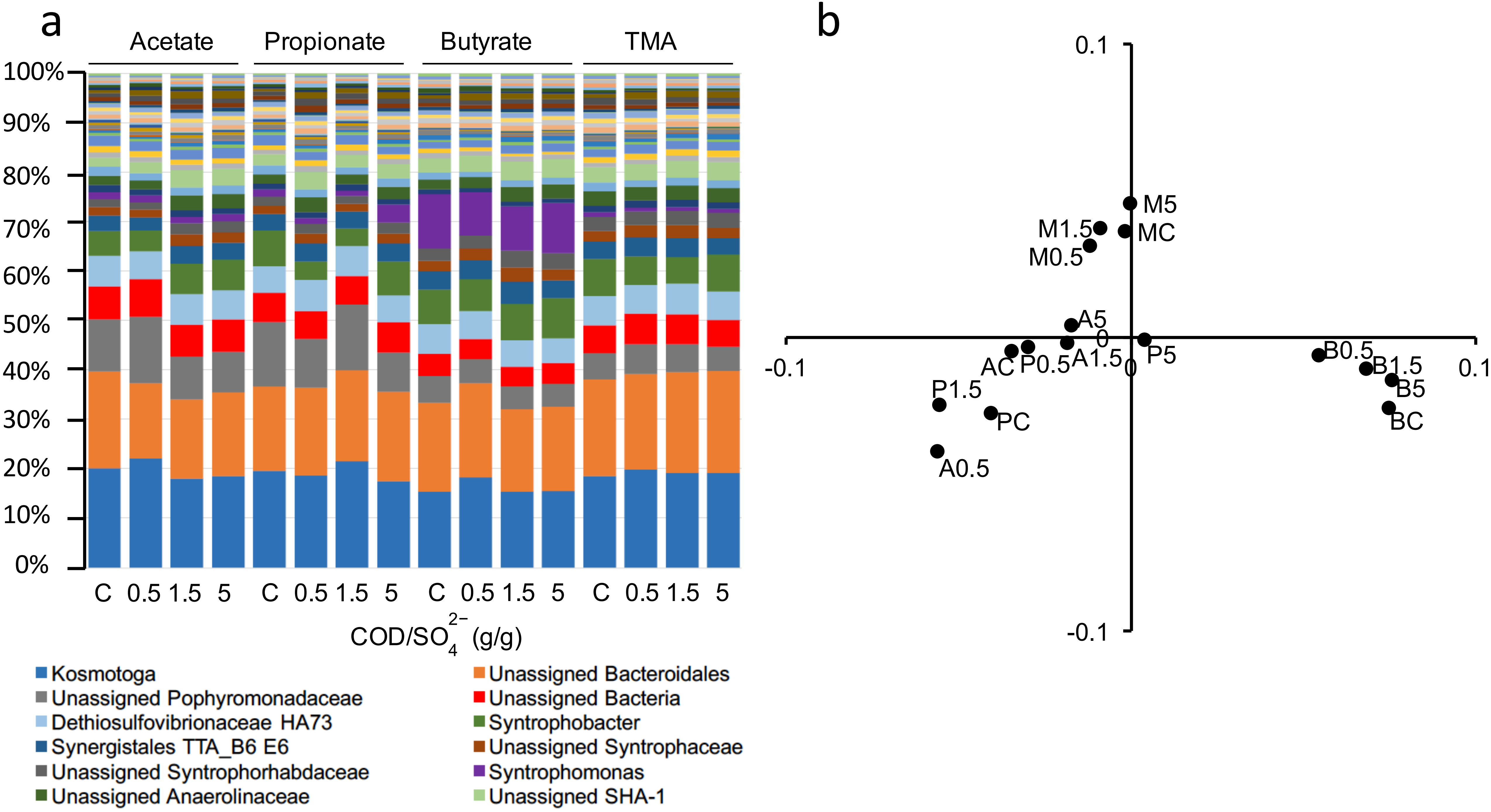
*TMA theoretical methane yield for 450 µmols of substrate was 1012.5 µmols, actual theoretical is based on total TMA consumed (70-73% of the calculated yield).


a
Methane Production (µmole)
0
200
400
600
800
1000
1200
1400
Theoretical methane production without sulfate
C
0.5
1.5
5
Acetate
Propionate
Butyrate
Trimethylamine
b
% Sulfate removal
0
10
20
30
40
50
60
70
80
90
100
Acetate
Propionate
Butyrate
Trimethylamine

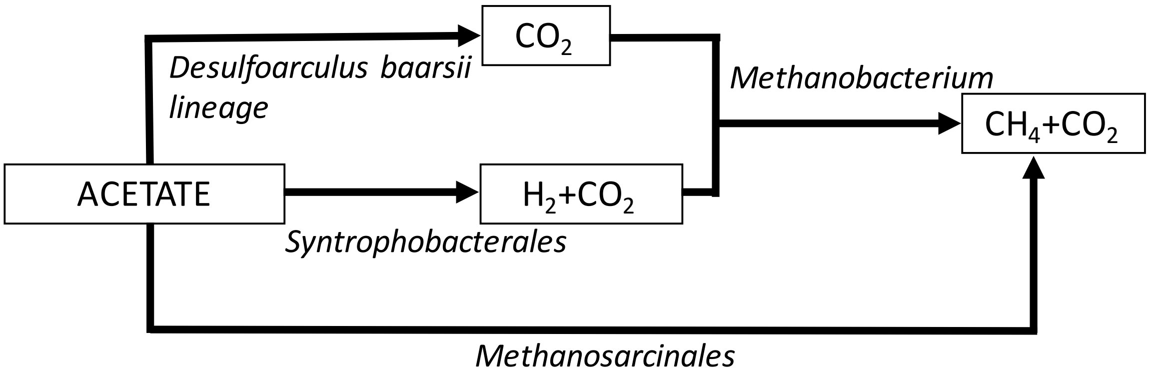

a
Acetate
Propionate
Butyrate
TMA
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C 0.5 1.5 5 C 0.5 1.5 5 C 0.5 1.5 5 C 0.5 1.5 5
COD/SO_4^{2-} (g/g)
Methanobacterium
Unassigned mcrA
Unassigned Methanosarcinales
Methanolinea
Methanomethylovorans
Unassigned Methanosarcinaceae
b
0.12
0.06
0
-0.06
-0.12
-0.3
0
0.3
B5
B1.5
PC
B0.5
BC
P0.5
MC
M0.5
M5
P1.5
AC
A0.5
M1.5
P5
A5
A1.5


a
Acetate
Propionate
Butyrate
TMA
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C
0.5
1.5
5
C
0.5
1.5
5
C
0.5
1.5
5
C
0.5
1.5
5
COD/SO_4^{2-} (g/g)
Methanosaeta
Unassigned Archaea
Methanomassiliicoccus
Methanobacterium
Unassigned Methanobacteriales WSA2
Methanomethylovorans
Unassigned MCG pGrfC26
b
0.02
-0.02
-0.05
0
0
0.05
A0.5
P1.5
P0.5
AC
MC
P5
PC
BC
A5
M5
M1.5
M0.5
B0.5
A1.5
B5
B1.5

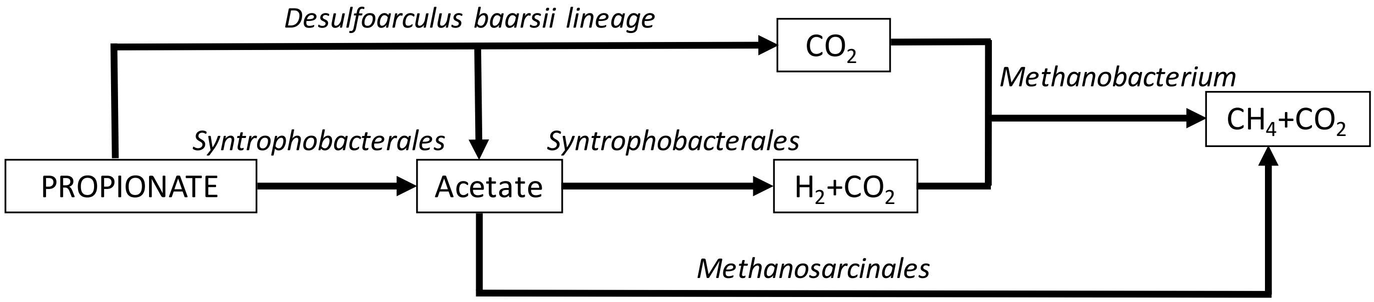

a
Acetate
Propionate
Butyrate
TMA
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C 0.5 1.5 5
C 0.5 1.5 5
C 0.5 1.5 5
C 0.5 1.5 5
COD/SO$_4^{2-}$ (g/g)
Desulfovibrionaceae
Desulfoarculus baarsii lineage
Other Deltaproteobacteria
Other Deltaproteobacteria supercluster
Other FACA group sensu lato
b
0.06
0.03
0
-0.03
-0.06
-0.3
0
0.3
PC
A1.5
P5
M5
B1.5
B5
BC
A5
MC
AC
A0.5
M1.5
P1.5
M0.5
P0.5
B0.5


a
Acetate
Propionate
Butyrate
TMA
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
C
0.5
1.5
5
C
0.5
1.5
5
C
0.5
1.5
5
C
0.5
1.5
5
COD/SO$_4^{2-}$ (g/g)
Kosmotoga
Unassigned Bacteroidales
Unassigned Pophyromonadaceae
Unassigned Bacteria
Dethiosulfovibrionaceae HA73
Syntrophobacter
Synergistales TTA_B6 E6
Unassigned Syntrophaceae
Unassigned Syntrophorhabdaceae
Syntrophomonas
Unassigned Anaerolinaceae
Unassigned SHA-1
b
0.1
-0.1
0
0
-0.1
0.1
M5
M1.5
MC
M0.5
A5
A1.5
P5
AC
P0.5
B0.5
B1.5
B5
BC
P1.5
PC
A0.5

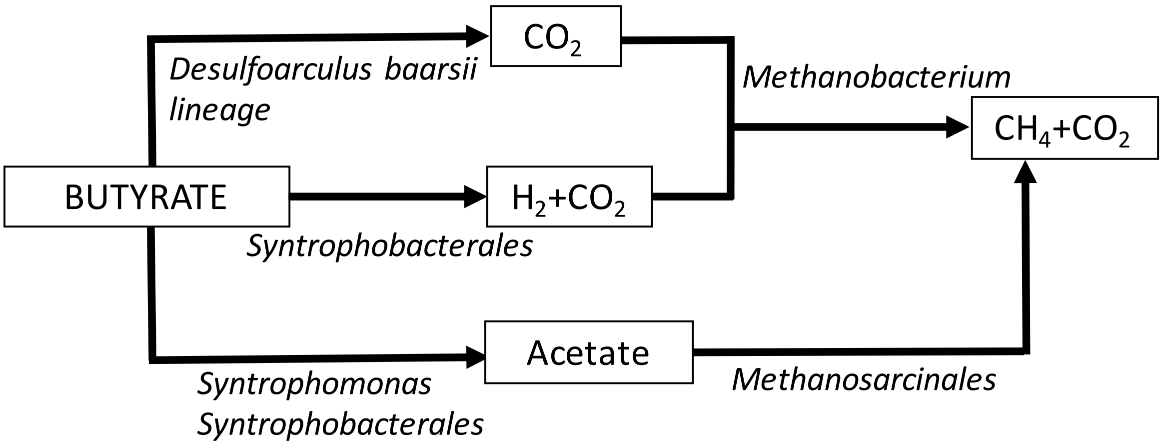
a


CO$_2$
Desulfoarculus baarsii lineage
Methanobacterium
CH$_4$+CO$_2$
ACETATE
H$_2$+CO$_2$
Syntrophobacterales
Methanosarcinales

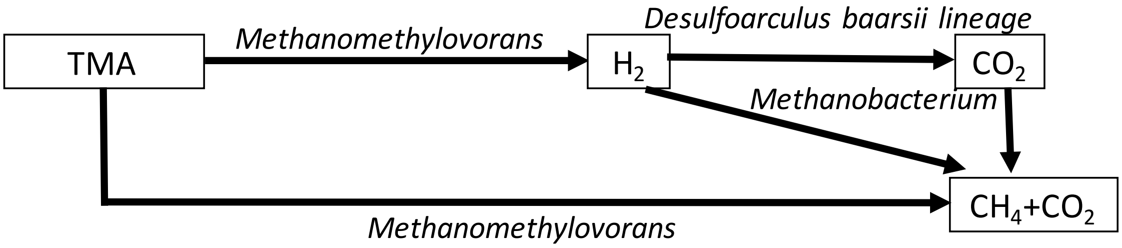
b


Desulfoarculus baarsii lineage
CO$_2$
Methanobacterium
CH$_4$+CO$_2$
Syntrophobacterales
Syntrophobacterales
PROPIONATE
Acetate
H$_2$+CO$_2$
Methanosarcinales

c


CO$_2$
Desulfoarculus baarsii lineage
Methanobacterium
CH$_4$+CO$_2$
BUTYRATE
H$_2$+CO$_2$
Syntrophobacterales
Acetate
Syntrophomonas
Syntrophobacterales
Methanosarcinales

d


Desulfoarculus baarsii lineage
TMA
Methanomethylovorans
H$_2$
CO$_2$
Methanobacterium
CH$_4$+CO$_2$
Methanomethylovorans